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TITLE: Neuroprotective Strategies for the Treatment of Blast-Induced Optic Neuropathy

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14. ABSTRACT Traumatic optic neuropathy is a rare but devastating injury that can result from blunt force or explosive blast. Presentation can be delayed by several weeks and patients ultimately lose vision completely in the affected eye. Unfortunately, in the military, damage is often bilateral. We use a mouse model of closed globe trauma to induce indirect traumatic optic neuropathy in order to test underlying mechanisms with the goal of identifying therapies for this currently untreatable blinding condition. Our work on this grant to date has identified sterile neuroinflammation as a key pathway involved in neurodegeneration after trauma. We have also packaged EPO-R76E into nanoparticles for intraocular delivery, after demonstrating that intraocular delivery is likely to be more efficacious than systemic delivery. We are currently treating mice with galantamine after trauma and will examine alterations in the amacrine cells and ganglion cells as well as therapeutic outcome measures including electroretinogram, visual evoked potential, and optical coherence tomography. <u>Finally, a pre-application to the DoD for a clinical study was invited for a full submission.</u>					
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1.Introduction: A major limiting factor to the development of treatments for indirect traumatic optic neuropathy has been the absence of a suitable animal model for: (1) mimicking the initial injury; and (2) tracking secondary degeneration. We have addressed this limitation in an innovative way, by developing an experimental system that models ocular blast injury.² This system recapitulates many of the same injuries detected in Service Members with blast-induced ocular trauma, including retinal detachments, optic nerve atrophy, and vision loss.^{1,3}

We will assess the efficacy of two therapeutic agents in our model of blast-induced traumatic optic neuropathy. Our model causes early oxidative stress, neuroinflammation and inner retinal dysfunction followed by decreased vision and optic nerve degeneration.¹⁻³ This suggests that degeneration of the retinal ganglion cell (RGC) axons in the optic nerve is a secondary event. Secondary degeneration of downstream neurons is well described in the central nervous system (CNS) after trauma.⁴⁻⁷ One such example within the visual system is degeneration in the lateral geniculate nucleus after lesion of the optic nerve or ocular hypertension.^{11, 12} Therefore, **our study has implications for neurodegenerations from trauma extending beyond optic neuropathy.**

2. Keywords:

retinal ganglion cell (RGC), traumatic optic neuropathy, inflammasome, erythropoietin (EPO), electroretinogram (ERG), visual evoked potential (VEP)

3. Accomplishments:

What were the major goals of the project?

Aim 1: Elucidate the cellular mechanisms underlying visual dysfunction after blast. *We will test the working hypothesis that blast activates pyroptosis in starburst amacrine cells causing decreased signaling to the dsRGCs, which leads to dendritic pruning and axon degeneration in the dsRGCs.*

Major Task 1: Obtain approval for mouse studies.

Major Task 2: Assess if pyroptosis is activated after blast. Months 5-12

Major Task 3: Assess if signaling from starburst amacrine cells is altered by blast. Months 12-14

Major Task 4: Determine if the dendritic trees of the dsRGCs are altered by blast. Months 5-24

Aim 2: Assess the efficacy of galantamine in preventing neurodegeneration secondary to blast. *We will test the hypothesis that galantamine will restore signaling to the dsRGCs thus preventing their degeneration after blast.*

Major Task 1: Assess vision in treated and control blast mice. Months 17-24

Major Task 2: Assess histology of treated and control blast mice. Months 20-28

Major Task 3: Quantify neurochemical changes in the retina. Months 20-21

Aim 3: Assess if reduction in neuroinflammation and oxidative stress by EPO-R76E prevents neurodegeneration secondary to blast. *We will test the working hypothesis that EPO-R76E will protect against blast-induced optic neuropathy by limiting oxidative stress and neuroinflammation.*

Major Task 1: Assess vision in treated and control blast mice. Months 28-34

Major Task 2: Assess histology of treated and control blast mice. Months 30-36

Major Task 3: Quantify EPO-R76E levels. Months 30-32

What was accomplished under these goals?

1) Major Activities:

A. We obtained approval for the mouse studies from Vanderbilt University and the Department of Defense.

B. We performed initial studies to compare a single hit to the more clinically relevant multiple hit paradigm. As a result of these studies we have converted to a multiple hit paradigm for the proposed studies.

C. We performed the Aim1, Task 1 studies with the exception of the immunolocalization studies due to antibody challenges.

D. We purchased and collected baseline data with a new Diagnosys LLC system that allows simultaneous recording of the electroretinogram (including oscillatory potentials) and visual evoked potentials. It also is able to perform pattern electroretinogram stimulation. This will give us simultaneous measurements of light evoked responses from the photoreceptors, bipolar cells, amacrine cells, ganglion cells, and visual cortex.

E. We published a peer-reviewed manuscript demonstrating that intraocular delivery of EPO-R76E is likely to be safer and more effective than systemic delivery. Delivery of adeno-associated virus into the eye results in long-term gene expression, which may not be needed in the context of trauma. Thus, we have formed a collaboration with Dr. Craig Duvall and are producing nanoparticles that delivery EPO-R76E.

2) Specific Objectives:

A) To obtain mouse research approval.

B) To determine if sterile inflammation is activated in the retina after ocular trauma.

C) To purchase and begin breeding *Drd4.eGFP* mice, which express eGFP in the directionally selective retinal ganglion cells (dsRGCs).

3) Significant Results:

A) We assessed levels and localization of multiple inflammasome pathway proteins in wild-type mice maintained on a control diet and exposed to blast (Figure 1). We detected an increase in the number of cells and total levels of the caspase that mediates this pathway (caspase 1; Figure 1A-D). Further, we detected an increase in the ratio of cleaved to total caspase 1 indicating activation of this enzyme after trauma (Figure 1D). The two major by-products of caspase 1 activation are IL-1 β and IL-18. We detect an increase in both (Figure 1E, G, H). Finally, similar to the results from the ketogenic study, we detect an increase in IL-1 α (Figure 1I). IL-1 α is active in both its pro- and cleaved forms and thus could be the initiator of the response. As shown in Figure 1J, oxidative stress can also feed into this pathway thus the antioxidant studies are still very relevant. Activation of this pathway can lead to cell death by over activation of the pannexin channel, leading to pyroptosis (Figure 1J). We are exploring potential therapeutic interventions for this pathway including treatment

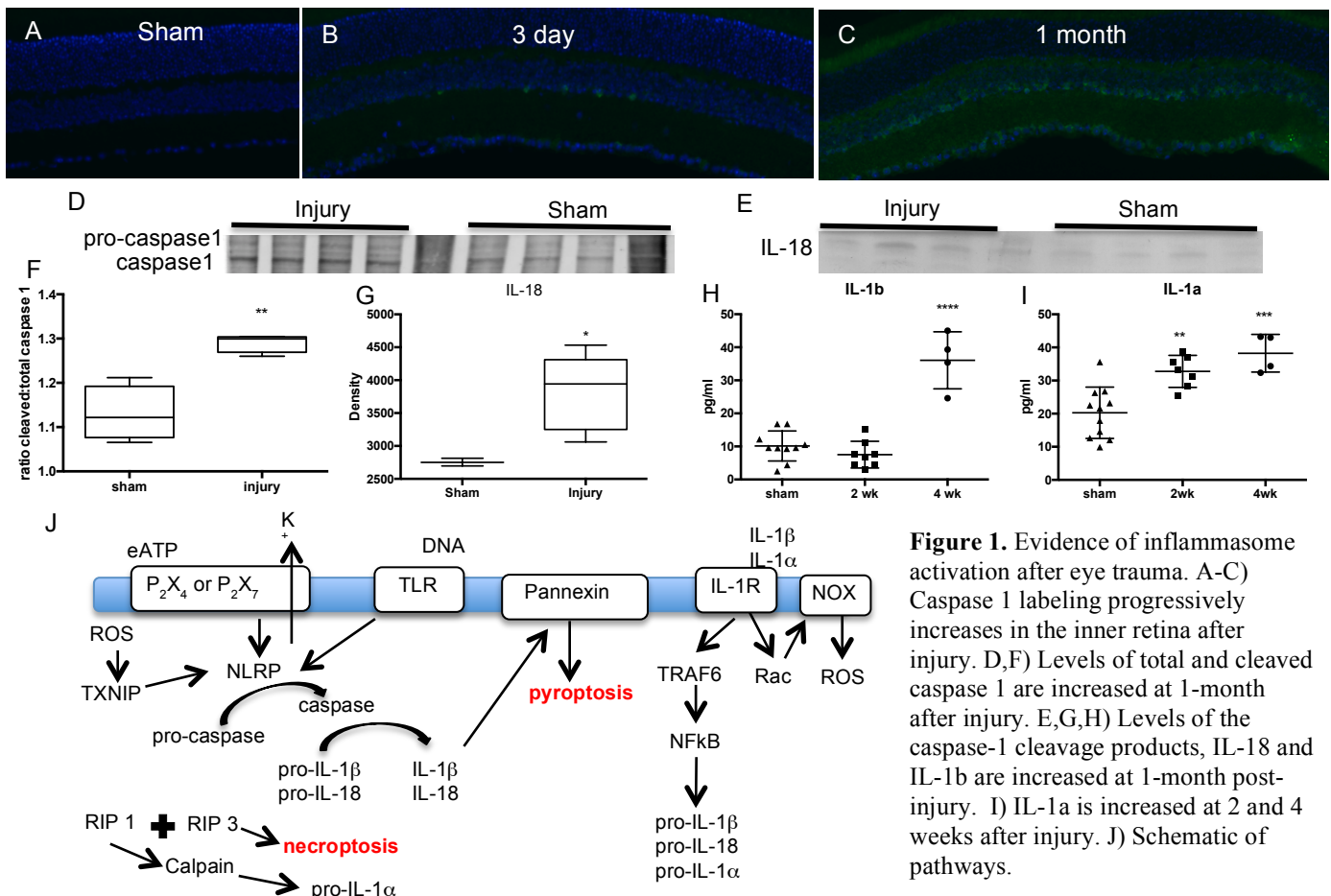


Figure 1. Evidence of inflammasome activation after eye trauma. A-C) Caspase 1 labeling progressively increases in the inner retina after injury. D,F) Levels of total and cleaved caspase 1 are increased at 1-month after injury. E,G,H) Levels of the caspase-1 cleavage products, IL-18 and IL-1 β are increased at 1-month post-injury. I) IL-1 α is increased at 2 and 4 weeks after injury. J) Schematic of pathways.

with IL-1 receptor (IL-1R) antagonist since both IL-1 α and IL-1 β act through the IL-1R. We are producing a recombinant adeno-associated virus that we will use to provide sustained delivery of the IL-1 receptor antagonist in the retina.

B) Since the caspase 1 labeling overlaps with acetylcholinesterase positive cells, we measured ACh levels at 1 month post-blast to determine if levels were decreased. No change in total ACh levels was detected. We will repeat the experiment at 3 months post-blast, when we detected a decrease in GABA levels (these cells co-release ACh and GABA). This study is ongoing and we expect results by the end of December.

C) We performed RNAseq and nanostring analysis on sham and blast retinas to determine if there were changes in mRNA or microRNA that might inform mechanism. We detected a 3-fold decrease in mRNA encoding prostate stem cell antigen (PSCA), a member of the Lynx proteins, is an antagonist of the $\alpha 7$ nicotinic ACh receptors and over-expression is neuroprotective in ciliary ganglia. Notably, the starburst amacrine cells (caspase 1 positive after trauma) signal onto the directionally selective retinal ganglion cells (dsRGCs) with GABA and ACh and the ds RGCs contain $\alpha 7$ nicotinic ACh receptors at post-synaptic locations on their dendrites. We are performing RT-PCR to determine if PSCA is expressed in retina. If it is, we will use real-time PCR to quantify if there is a change in levels between sham and blast retinas as suggested by the RNAseq results. We also detected 2-fold increases in microRNAs that inhibit retinal angiogenesis and/or are important in development/induction of retinal pigment epithelial cells.

D) We are breeding the Drd4.eGFP mice to expand the colony and backcross. These mice express green fluorescent protein in the dsRGCs and thus will allow us to quantitatively assess alterations in the dendritic trees of these cells. We are also exploring the purchase of Neurolucida, a software that performs automated, quantification of neurite length, branching, thickness, and other measurements.

E) Finally, we have performed repeat blast studies showing that 3 15psi blasts is similar or worse than a single 26psi blast. Since most service members are repeatedly exposed to blasts and even in a single blast they can be hit by blast waves multiple times, we have updated our trauma regimen to two back-to-back 15psi blasts repeated three times at 24hr intervals.

F) We have purchased a system from Diagnosys LLC that allows us to simultaneously measure the electroretinogram (ERG), and visual evoked potential from the same mouse. We are also now able to perform pattern ERGs (Figure 2). The 3-month long pinworm treatment of all mice in our facility has finally ended and we have performed all of the baseline assessments for this study (Figure 2).

G) We recently published showing that delayed, but not early, treatment with EPO is protective to the retina and optic nerve after trauma (ref). This was also true for systemic gene delivery of EPO-R76E. The lack of efficacy early on appears to be related to the slight, but still significant, increase in hematocrit, resulting in increased oxidative stress in the retina. This study told us two things: 1) Intraocular delivery of EPO may be most effective, and 2) therapy can be delayed for 3 weeks after injury and still yield therapeutic benefit.

H) With the assistance of the Vanderbilt Protein and Antibody Resource we produced 98% pure human EPO-R76E and, in collaboration with Dr. Craig Duvall, produced PPS nanoparticles that carry this protein. Using a standard protein assay, we estimate that we packaged approximately 1-2 μ g EPO-R76E per particle. We expect to be able to deliver sufficient nanoparticles into the eye to deliver up to 200ng EPO-R76E. We estimate

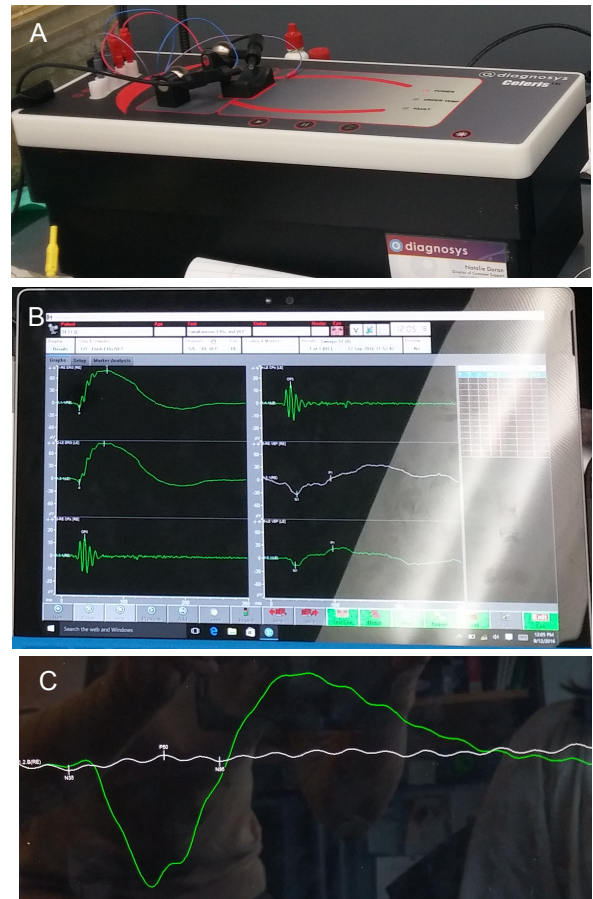


Figure 2. The new Diagnosys LLC system (A) that allows simultaneous measurement of the ERG (B1), oscillatory potentials (B2), and VEP (B3) from both eyes/visual cortices. The system can also perform a full-field pattern ERG (C).

to only need continuous delivery of 0.02ng EPO-R76E for therapeutic benefit based on work from our laboratory and other publications. Notably these nanoparticles have innate antioxidant properties, which should enhance the therapeutic efficacy of this approach. We are currently performing analysis of the release kinetics of EPO-R76E from these particles to determine if we will meet the 1% release that we need and to determine the likely longevity of the particles *in vivo*. We expect that they will be effective for approximately 3 months. Once we have determined the release kinetics we will test the efficacy of the nanoparticles when injected into the vitreous after ocular trauma.

4) Other Achievements:

Finally, as part of our Translation Plan, a pre-application was submitted to the Department of Defense Psychological Health/Traumatic Brain Injury Research Program for the Complex Traumatic Brain Injury Rehabilitation Research Award. The premise of the proposal was that sensitive and reliable quantitative tests of vision are needed. Currently patient complaints are not matched by quantitative clinical assessments. Until tests are identified clinical trials with any potential therapeutic cannot go forward due to the lack of outcome measures. The full proposal will be submitted at the end of November.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

We recently published the paper on the effect of systemic administration of EPO in the Journal of Optometry and Visual Science. No other results have been published to date.

What do you plan to do during the next reporting period to accomplish the goals?

We are breeding the Drd4.eGFP mice, but it is unlikely we will be able to gather data from these mice this year due to the need to backcross.

We are going to assess ACh levels at 3 months after blast.

We just started a cohort of mice that received galantamine beginning right after blast exposure.

We are characterizing the EPO-R76E containing nanoparticles and, if all looks fine, we will test them *in vivo* in our model.

4. Impact:

What was the impact on the development of the principal discipline(s) of the project?

Our discovery that the timing of delivery of erythropoietin is critical may contribute significantly in the interpretation of previous and ongoing clinical trials and animal studies. Our data suggests that direct delivery of erythropoietin into the CNS may be more effective by avoiding the harmful side-effect of even a slight increase in red blood cell production caused by systemic delivery. Finally, our data shows that treatment can be delayed by as much as three weeks after the injury and still protect the retina and optic nerve. This is surprising since the mantra has been to treat within 24hours. In combination this study suggests that oxidative stress contributes significantly to neurodegeneration after ocular trauma, early intervention is not always best, and that the therapeutic window of opportunity is much longer than previously thought.

We have identified an increase in sterile inflammation (inflammasome) as one of the earliest markers of damage in the retina after ocular trauma. There are several druggable targets in this pathway that could be therapeutically efficacious for the treatment of indirect traumatic optic neuropathy. We are beginning to explore this possibility.

What was the impact on other disciplines?

We recently published that systemic treatment with EPO could be detrimental to the retina after trauma. We showed that this was due to an increase in hematocrit, which increased the ongoing oxidative stress in the retina after injury. This is likely relevant to the traumatic brain injury field. EPO is currently in Phase III trials

for traumatic brain injury, but animal studies are conflicting. It is feasible that researchers and clinicians are treating with inappropriate dose or timing.

What was the impact on technology transfer?

In the current study we have packaged a form of EPO with attenuated erythropoietic activity (EPO-R76E) into nanoparticles to provide sustained but reversible treatment *in vivo*. Successful completion of this project may well yield a clinically translatable product. Both the nanoparticles and EPO-R76E are novel. Our collaborator, Dr. Duvall, has connections to pharmaceutical companies whom he has licensed other nanoparticles to in the past, so we have a path to the market and to the clinic.

What was the impact on society beyond science and technology?

Nothing to Report.

5. Changes/Problems:

Changes in approach and reasons for change:

In military and sports head trauma with resulting functional damage due to repeat injury either during the same event or in repeated events is more common than a single isolated traumatic injury. For example, in a single explosive blast a person can be exposed to damage from the blast wave and damage from the head striking another object and these two physical events can occur multiple times during the single blast. In addition, most blast injured service members were exposed to multiple blasts including as many as a hundred. Thus, it is more clinically relevant to model multiple traumatic events that are non-injurious on their own, but repeat exposure results in damage. We tested this in our model and have identified a regimen of 2 back-to-back 15psi blasts repeated daily for 3 days as an optimal blast scenario more representative of real world experiences. All studies in this project will be performed using this paradigm.

In Specific Aim 3 we proposed to use recombinant adeno-associated virus to deliver EPO-R76E as a proof of concept with the goal in the Transitional Plan of developing nanoparticles for future clinical trials. We were fortunate to develop a collaboration with Dr. Craig Duvall, Vanderbilt University, who is an expert in development of nanoparticle mediated drug delivery. Together we have already produced the first set of nanoparticles. If the release kinetics is promising (experiments on going), we will use these nanoparticles in Aim 3 instead of the adeno-associated virus in order to more quickly translate to the clinic.

Actual or anticipated problems or delays and actions or plans to resolve them:

The Vanderbilt University Medical Center animal facility decided to treat all mice for pinworms. Their treatment included hydrogen peroxide, which induces oxidative stress. We have shown that oxidative stress is already increased in the retina after ocular trauma and exacerbation of oxidative stress (by increased delivery of oxygen and iron via erythropoiesis, for example) causes greater retinal cell death. Thus, the pinworm treatment had a likelihood of affecting our studies. So, we had to stop all mouse studies for the duration of the treatment and for an additional month afterwards to assure that the mice were healthy and “normal” again. As a result we lost 4 months worth of work.

Two laboratory staff members left unexpectedly and without warning. Neither one gave notice – they were gone the day they informed me by email and they never returned. In both cases it was for personal reasons, however, regardless of the reason it left us struggling to manage the laboratory experiments. A new person has just joined the lab and has been trained on the major outcome measures used in the laboratory. In addition, I currently have two job postings and am screening through the candidates to identify new hires.

My graduate student, Courtney Bricker-Anthony just successfully defended her dissertation. She is making revisions to the dissertation document and will graduate at the end of 2016. I am actively looking to recruit post-doctoral fellows and/or graduate students into the lab.

Changes that had a significant impact on expenditures:

Due to the lack of appropriate staff, we are behind in breeding and genotyping the Drd4.eGFP mice. In addition, expenditures in personnel are lower than it should be as we are in need of personnel to perform the studies. As mentioned above we are in the process of identifying qualified candidates to hire.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:

Nothing to Report.

6. Products:

Publications, conference papers, and presentations:

Bricker-Anthony C, D'Surney L, Lunn B, Jo M, Bernardo-Colon A, Hines-Beard J, Rex TS (2016) Erythropoietin can both promote and prevent retinal damage after eye trauma. *J Optom Vis Sci. (Trauma special issue)* Epub

Rex TS, Bricker-Anthony C (2016) Erythropoietin can both promote and prevent retinal damage after eye trauma. National Neurotrauma Symposium. J Neurotrauma Suppl.

Rex TS, Bricker-Anthony C (2016) Mechanisms and therapy for blast-induced eye trauma. ISER, Exp Eye Res. Suppl.

Website or other internet site:

Nothing to Report

Technologies or techniques:

Nothing to Report

Inventions, patent applications, and/or licenses:

We have developed antioxidant nanoparticles that are loaded with EPO-R76E. We are now testing the release kinetics and will begin in vivo studies shortly thereafter if all goes well. The nanoparticles are biocompatible so this is a product that has the ability of being licensed and used in the clinic in the future.

7. Participants & Other Collaborating Organizations:

What individuals have worked on the project?

Name:	Tonia S. Rex
Project Role:	PI
Nearest person month worked:	5
Contribution to Project:	Supervised all activities, designed studies, trained lab members, published and presented research.

Name:	Alexandra Bernardo
Project Role:	RA III
Nearest person month worked:	12
Contribution to Project:	Lead researcher for all experiments. Designed and performed experiments, trained Cinsley.

Name:	Lorraine Kasmala
Project Role:	RA III
Nearest person month worked:	4
Contribution to Project:	Performed some animal experiments, performed histology, ordered reagents.

Name: Cinsley Gentillon
Project Role: RA II
Nearest person month worked: 4
Contribution to Project: Assisted Alexandra

Name: Marcus Colyer
Project Role: Consultant
Nearest person month worked: N/A
Contribution to Project: During my first visit to meet with Dr. Colyer, I gave a lecture to the Ophthalmology Department and met with the Ophthalmologists individually. I also was introduced to and given a tour of the National Intrepid Center of Excellence and met with the Director of the program as well as the Neuro-optometrist there. During that visit we learned of the satellite Intrepid Centers and learned that one is housed at Ft. Campbell, which is only one hour from Vanderbilt University. Dr. Colyer has also traveled to Vanderbilt University Medical Center, gave a Grand Rounds lecture to the Department of Ophthalmology and another lecture to the Residents. We traveled together to Fort Campbell, KY, where we met with the Ophthalmology Department, Optometrists, and the Director of the Army Intrepid Spirit and Sleep Medicine Program. That meeting was critical for the development of a relationship between me and the clinicians and researchers at Ft. Campbell that has led to submission of a pre-application to the Department of Defense for a clinical study on complex traumatic brain injury patients with self-reported visual and/or auditory complaints. We were just invited to submit a full proposal. In a subsequent visit to Walter Reed National Military Medical Center, I gave a presentation on blast physics and biomechanics during the Tri-Service Ocular Trauma Course for military Ophthalmology residents at the Uniformed Services University. I was also able to learn from the fantastic lectures given by military Ophthalmologists and see the lab. This interaction helped me to gain a greater understanding of the clinical picture and to realize even more so the reality that these patients underwent several traumas – blunt and blast – and thus, a repeat hit paradigm is more clinically relevant. We have since switched to this paradigm for our studies. I will be participating in the Tri-Service Ocular Trauma Course again this year.

Name: Craig Duvall
Project Role: Collaborator
Nearest person month worked: N/A
Contribution to Project: Developed nanoparticles and packaged EPO-R76E into them.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. Special Reporting Requirements:

None.

9. Appendices:

See attached updated Quad Chart.

Neuroprotective strategies for the treatment of blast-induced optic neuropathy

MR141315

W81XWH-15-1-0559



PI: Tonia S. Rex

Org: Vanderbilt University Medical Center

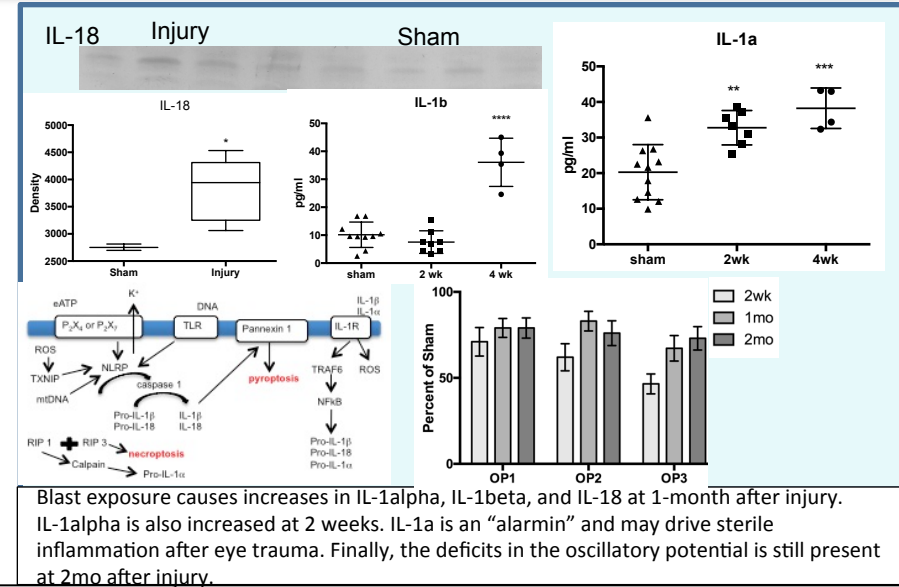
Award Amount: \$1.5 million

Study/Product Aim(s)

- We hypothesize that blast-induced optic nerve degeneration and vision loss is due to oxidative stress and neuroinflammation, which causes cholinergic neuron dysfunction.
- Aim 1: We will test the working hypothesis that blast activates inflammation-mediated cell death in the cholinergic amacrine cells and leads to decreased signaling to the direction-selective retinal ganglion cells and degeneration of their axons.
- Aim 2: We will test the working hypothesis that restoration of signaling to the retinal ganglion cells by treatment with galantamine will preserve the optic nerve and vision after blast.
- Aim 3: We will test the working hypothesis that a non-erythropoietic form of erythropoietin (EPO-R76E) will block oxidative stress and neuroinflammation and preserve the optic nerve and vision after blast.

Approach

We will use our model of blast induced optic neuropathy to assess the efficacy of galantamine and erythropoietin. We will quantify relevant neurotransmitters, oxidative stress, neuroinflammation, axon transport, histology, and vision.



Timeline and Cost

Activities	CY	15	16	17	
Specific Aim 1					
Specific Aim 2					
Specific Aim 3					
Estimated Budget (\$K)		\$504	\$506	\$490	

Goals/Milestones

CY16 Goal – Determine the role of inflammation-mediated cell death and astrocyte dysfunction on blast induced vision loss and axon degeneration.

- ☐ Quantify levels of ACh after blast
- ☐ Measure the dendritic fields of the retinal ganglion cells

CY17 Goal – Determine the efficacy of galantamine

- ☐ Quantify vision, and optic nerve histology in treated and control mice.
- ☐ Quantify secondary outcome measures in treated and control mice.

CY18 Goal – Determine the efficacy of EPO-R76E on blast induced optic neuropathy.

- ☐ Quantify vision, and optic nerve histology in treated and control mice.
- ☐ Quantify secondary outcome measures in treated and control mice.

Budget Expenditure to Date

Projected Annual Expenditure: \$504,000

Actual Annual Expenditure: \$304,999.75

Updated: 6/24/16